Laboratory of Microbiology

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Anaerobic Biodegradation of Benzene in Contaminated Soils

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1 Summary

Anaerobic bioremediation is most attractive whenever anaerobic conditions prevail in a polluted soil site. Thus far, anaerobic bioremediation techniques for soils polluted with mobile aromatic hydrocarbons are not applied. The bottleneck in the application of anaerobic techniques is the supposed poor anaerobic biodegradability of benzene. However, anaerobic degradation of benzene under various redox conditions has been described, but the microorganisms involved are not known. Only very recently the first anaerobic benzene-degrading microorganism was described (7). However, the optimal physiological conditions for anaerobic benzene degrading microorganisms and the biodegradation pathway are unknown. Such knowledge is essential to apply anaerobic bioremediation techniques for soils polluted with BTEX compounds.

The aim of this research project is to get insight into the occurrence of anaerobic benzene oxidation in polluted sites by studying microorganisms involved in anaerobic benzene oxidation. Therefore, the aim is to isolate or enrich anaerobic benzene degrading bacteria and study their physiological and phylogenetic properties. This knowledge can be used to determine and stimulate anaerobic benzene oxidation in the field.

The acquired knowledge will lead to the development of a novel bioremediation technology and may also result in methods to demonstrate and monitor anaerobic benzene degrading bacteria in polluted soils. This project will be linked to ongoing projects, including the SKB project "Flebo, phase II", a collaboration of Oranjewoud, TNO-MEP, TNO-NITG and Oosterhof Holman and the TNO-MEP project "Demonstration of bioaugmentation of benzene degrading activity" in collaboration with NAM, IWACO and Oosterhof Holman. In addition, this research will be linked to compound specific stable carbon isotope analysis of groundwater from polluted sites.

This first progress-report will summarise the literature concerning the anaerobic biodegradation of benzene. The batch experiments that are being conducted will be briefly described. Anaerobic benzene degradation is being tested under various redox conditions with different inocula and with pure cultures. Unfortunately, all the results till now do not show any anaerobic benzene degradation. However, long lag phases are found in literature and therefore measurements will be continued. Future, research will focus more on available anaerobic benzene degrading enrichments. Furthermore, continuous column experiments will be conducted in order to see the effect of continuous dosage of benzene and refreshing the liquid medium. Other approaches that will be evaluated are different polluted inoculum sources and electron acceptors (anthraquinonedisulfonate (AQDS) and manganese(IV)).

2 Anaerobic Biodegradation of Benzene

For a long time it was thought that benzene is persistent under anaerobic conditions. Only recently anaerobic biodegradation of benzene has been reported. Benzene can be oxidized with different electron acceptors for anaerobic respiration like iron(III), sulfate and nitrate. Furthermore, under methanogenic conditions benzene can be converted to methane and carbon dioxide. This paragraph gives an overview of the literature concerning the anaerobic biodegradation of benzene under the different terminal electron accepting processes (TEAP, Table 1). First, more focus will be given to the different TEAP under which anaerobic biodegradation was described. Second, isolated bacteria and enrichments will be discussed. Third, the anaerobic benzene biodegradation pathway will be discussed.

Table 1. Different TAEP processes under which anaerobic biodegradation of benzene occurred (3).

TEAP	**Overall energetic equation**		**Δ G° (kJ/mol)**
CO_2/CH_4	$4C_6H_6 + 27H_2O$	$9HCO_3^- + 15CH_4 + 9H^+$	- 116
SO_4^{2-}/H_2S	$4C_6H_6 + 15SO_4^{2-} + 12H_2O$	$24HCO_3^- + 15HS^- + 9H^+$	- 200
NO^{3-}/NO^{2-}	$C_6H_6 + 15NO_3^- + 3H_2O$	$6HCO_3^- + 15NO_2^- + 6H^+$	- 2020
NO^{3-}/N_2	$C_6H_6 + 6NO_3^-$	$6HCO_3^- + 3N_2$	- 2990
Fe^{3+}/Fe^{2+}	$C_6H_6 + 30Fe^{3+} + 18H_2O$	$6HCO_3^- + 30Fe^{2+} + 36H^+$	- 3070

Methanogenic benzene biodegradation
One of the first anaerobic benzene degradation studies was conducted under methanogenic conditions. Grbic-Galic and Vogel (1986, 1987) found anaerobic biodegradation of benzene under these conditions (10, 25). The degrading activity originated from a ferulic acid (aromatic compound) degrading sludge. The percentage of benzene degradation based on methane produced in unlabeled experiments and carbon dioxide in labeled production differed tremendously (Table 2). Therefore, it is questionable whether benzene was mineralized. However, experiments with ^{18}O labeled water showed incorporation of the ^{18}O labeled oxygen into phenol, a intermediate of benzene biodegradation. Further evidence for methanogenic biodegradation was found in later studies with polluted sediments and aquifers as inoculum (12, 28). Difference in lag phase can be explained by difference in exposure time to benzene of the inoculum.

Sulfate reduction coupled with benzene oxidation
Biodegradation of benzene under sulfate reduction conditions was often studied and found (Table 2). As source of microorganisms polluted marine sediments and estuarine and fresh water aquifers were used (9, 12, 19, 26). Addition of molybdate, an inhibitor of sulfate reduction, inhibited benzene degradation, showing that benzene oxidation is coupled to sulfate reduction (14).

In some experiments other aromatic compounds, like toluene and xylene, were added and were found to enhance biodegradation benzene (19). During column experiments with Ponca City aquifer, sulfate addition stimulated anaerobic biodegradation of benzene (27). Furthermore, addition of sulfate reducing enrichment culture to sediments from sulfate reduction zone of petroleum contaminated aquifer showed to have a positive influence on the benzene degradation (27).

Nitrate reduction
Recently, two bacteria were described that were able to couple nitrate reduction to benzene oxidation. Two strains, RCB and JJ, will be described further in this paragraph under "Isolate bacteria and enrichments" (7). First indication that benzene oxidation was coupled to nitrate reduction was found by Major et al. (17). However, no carbon dioxide measurements were done. Therefore, it was questionable whether benzene was completely mineralized. Burland and Ewards (1999) showed that benzene mineralization was coupled with nitrate reduction to nitrite (3).

Table 2. Summary of anaerobic benzene degradation that was published with different TEAP.

TEAP conditions	Percentage degraded	Concentration (mM)	Originated from	Lag phase (d)	Reference
CH_4/CO_2	6 [1] (50 [2])	1.5 - 30	Ferulic acid degrading sludge	16	(10)
CH_4/CO_2	82 [1]	0.05	aquifer sediment, MI	420	(12)
CH_4/CO_2	73 [2]	0.675	aquifer sediment, MI	360	(12)
CH_4/CO_2	53 [1]	???	aquifer Ponca City, OK	0	(28)
SO_4^{2-}/H_2S	90 [1]	0.20	Seal Beach, CA	70-100	(9)
SO_4^{2-}/H_2S	92 [1]	1.7	San Diego Bay, CA	55	(14)
SO_4^{2-}/H_2S	92 [3]	0.125	Guaymas Basin, Mexico	84	(18)
SO_4^{2-}/H_2S	78 [1]	0.050	aquifer sediment, MI	400	(12)
SO_4^{2-}/H_2S	76 [1]	0.057	Seal Beach, CA	120	(12)
SO_4^{2-}/H_2S	85 [2]	0.125	NY/NJ Harbor sediment	60	(12)
SO_4^{2-}/H_2S	101 [2]	0.100	Sleeping Bear Dunes National Lakeshore, Empire, MI	100	(20)
NO_3^-/N_2	95 [4]	0.038	Canada Force Base Borden, Ontario	-	(17)
NO_3^-/NO_2^-	92-95 [1]	0.150	Toronto, Ontario	30	(3)
NO_3^-/NO_2^-	92-95 [1]	0.150	Fresh water swamp, Perth, Ontario	30	(3)
NO_3^-/N_2	47 [1]	0.163	Strain JJ and RCB	0	(7)
Fe^{3+}/Fe^{2+}	86 [1] 97 [2]	0.608	Sediment Defense Fuel Center, Hanahan, SC	87-122	(16)
Fe^{3+}/Fe^{2+}	95 [5]	0.010	Sediment Defense Fuel Center, Hanahan, SC	25	(15)
Fe^{3+}/Fe^{2+}	46 [4]	0.125	NY/NJ Harbor sediment	100	(12)
Fe^{3+}/Fe^{2+}	100 [4]	0.003	Potomac River, Maryland	50	(12)
Fe^{3+}/Fe^{2+}	50 [1]	???	USGS Groundwater Toxic site, Bemidij, MN	0	(1)
Fe^{3+}/Fe^{2+}	90 [1]	0.050	aquifer Ponca City, OK	???	(5)

[1] Based on labeled $^{14}CO_2$ production
[2] Based on conversion of electron acceptor
[3] Based on benzene measurements also $^{14}CO_2$ detected
[4] Based on benzene measurements
[5] Test with other chelating substances

Iron reduction

The first indication that benzene was oxidized with the reduction of iron(III) was reported with addition of the iron chelating substance (making iron(III) more water soluble) nitrilotriacetic acid (NTA). Without NTA degradation of benzene did not occur. Benzene degradation rates increased by readdition of benzene (16). Other chelating substances like EDTA, N-methyliminodiacetic acid ethanol diglycine, humic acids and phosphates stimulted benzene oxidation coupled with iron(III) reduction (15). Studies with fresh water aquatic sediments from Potomac River indicated that additions of chelating substance for iron(III) reduction in not always required (5).

Isolated bacteria and enrichments

Recently, two strains RCB (Figure 1) and JJ were isolated that could degraded benzene anaerobically with nitrate as electron acceptor (7). Surprisingly, both strains were not isolated with benzene as electron donor. Strain RCB was isolated on 4-chlorobenzoate (0.5 mM) as electron donor and chlorate (10 mM) as electron acceptor. Inocula were taken from the Potomac River, Maryland. Strain JJ was isolated with 2,6-anthrahydroquinone disulphonate (5 mM) as electron donor and nitrate (5 mM) as electron acceptor. Acetate (0.1 mM) was added as carbon source. This strain was isolated from sediment collected from Campus Lake, Southern Illinois University. Phylogenetic analyses of both strains revealed that both strains are a member of the *Dechloromonas* subgroup in the ß subclass of the Proteobacteria. Based on the conversion of the electron acceptor (86% of nitrate consumed compared with of benzene degraded) it was concluded that benzene oxidation is coupled to nitrate reduction. Experiments conducted with ^{14}C labeled benzene led to the formation labeled carbon dioxide. This confirmed the benzene mineralization (7).

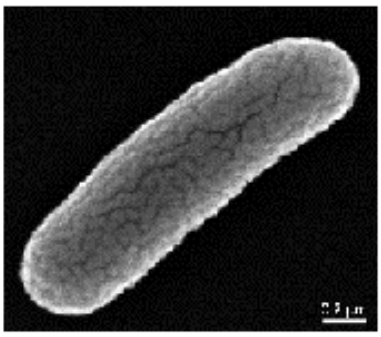


Figure 1. SEM micro-graph of strain RCB.

A sulfate reducing benzene mineralizing enrichment was characterized with different techniques. This stable and sediment-free, enrichment culture resisted repeated attempts of isolation. Molecular approaches such as traditional cloning and sequencing and a direct PCR fingerprinting method for 16S rRNA genes were used to determine the composition of the enrichment. Unfortunately, even long after exposure to benzene (over 3 years) and repeated dilutions of the original enrichment, the consortium still remained complex. Cloning and sequence analysis identified 12 unique small subunit rRNA genes. The genes belonged to different eubacterial phyla, including Proteobacteria, Cytophagales and Gram-positives (19) (Figure 2).

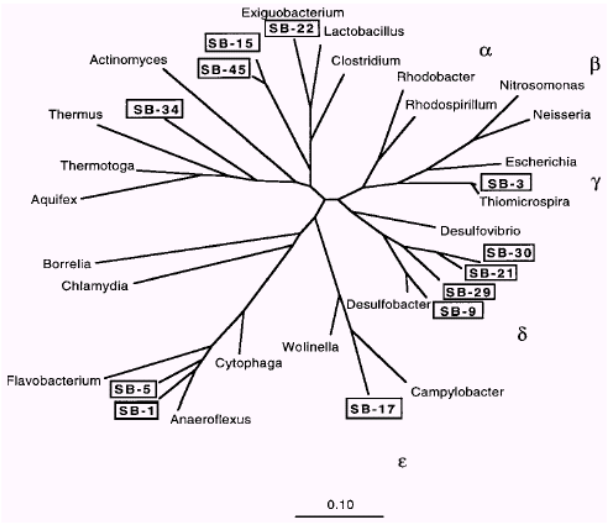


Figure 2. Phylogenetic tree showing the relationship of cloned sequences from the sulfidogenic benzene mineralising enrichment culture to the major taxa of the domain `bacteria` (19).

The microbial composition of a iron(III) reducing community that oxidizes benzene from a petroleum-contaminated aquifer of Bemidji was characterized and compared with iron(III) reducing uncontaminated sites via 16sRNA and DGGE fingerprinting (23). The polluted site contained many different Geobater strains well known for their ability to reduce iron(III).

Pathway elucidation

As already mentioned the first step in the biodegradation pathway of benzene under methanogenic conditions is the incorporation of oxygen from water yielding phenol. ^{18}O labeled phenol was found in cultures degrading benzene in ^{18}O water. Furthermore, in cultures fed with methanol and benzene phenol the major intermediate was cyclohexanone. Other intermediate that was detected was propionate (Figure 3) (10, 25).

An elegant way to investigate whether benzene is degraded via extracellular intermediates is by addition of possible intermediates together with ^{14}C labeled benzene. If a possible intermediate is added the production of labeled carbon dioxide is inhibited due to the presence of high amounts of these extracellular intermediates. This so-called isotope trapping method was preformed for several possible intermediates in a methanogenic benzene degrading enrichment. The results show that phenol, propionate and acetate are extracellular intermediates. Compounds that did not show inhibition of the carbon dioxide production were p-hydroxybenzoate, benzoate and butyrate indicating that these are no extracellular intermediates.

Another study, in which ^{13}C labeled benzene was used and intermediates were detected with GC/MS, showed that benzoate and phenol were intermediates under methanogenic conditions (4). Results revealed that the carboxyl group of benzoate was ^{13}C labeled while using 6 ^{13}C labeled benzene. This showed that carbon atom of benzene is used to carboxylate phenol or benzene. Due to the fact that p-hydroxybenzoate was not found in this study a direct carboxylation of benzene can be ruled out.

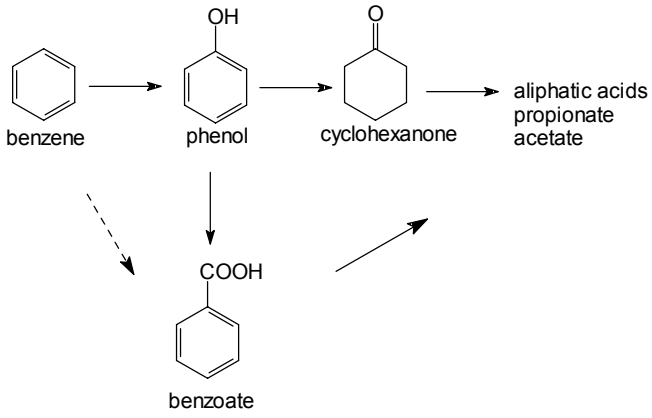
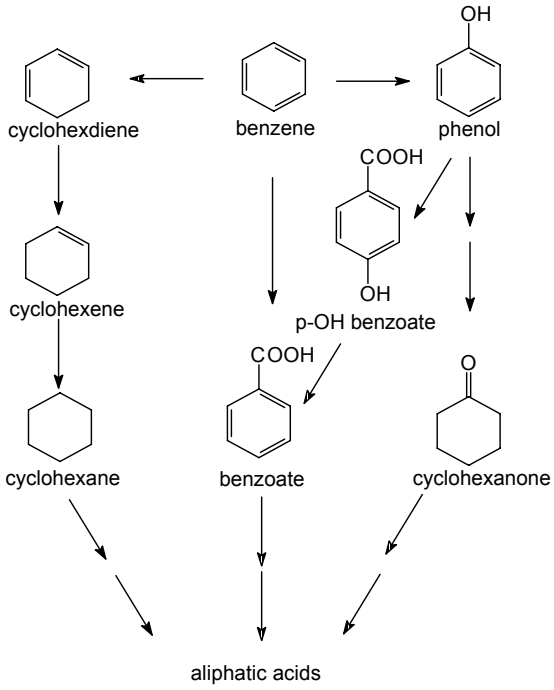


Figure 3. Intermediates found during methanogenic biodegradation of benzene.

Under sulfate reducing conditions no extracellular intermediates were found via the isotope trapping method, acetate, benzoate, p-hydroxybenzoate and cyclohexane were tested. This could indicate that under these conditions benzene is completely degraded by one organism (14). Furthermore, as already described above under methanogenic conditions, under sulfate reducing conditions phenol and benzoate were also found as intermediates (4). Again the benzoate had seven ^{13}C atoms indicating a carboxylation via a benzene carbon atom. Benzoate (D5) was also detected if deuterated (D6) benzene was used. However, (D5 or D6) phenol was not detected. Additions of ^{13}C-bicarbonate and ^{13}C-acetate showed that direct carboxylation of benzene is not the possible pathway for the formation of benzoate. Also the incorporation of the ^{13}C label was not found. This underscores the finding that the carbon atom of the carboxylgroup of benzoate is benzene itself (21).



Under iron(III) reducing conditions benzoate and phenol were again detected as intermediates (4). Benzoate was also formed via the carboxylation with a carbon atom of benzene.

Figure 4. Some possible anaerobic biodegradation pathways of benzene (benzoate, phenol, cyclohexanone, propionate and acetate were detected as intermediates)

3 Research and Results

Polluted sediments and other inoculations

A polluted sediment (1st Petrol Harbour, Amsterdam (PH)), soil (Van Velden Buren (VVB)) and biomass from a refinery (Repsol, Spain (Rep)) are used as inoculum to degraded benzene under anaerobic conditions. Anaerobic batches with mineral medium are inoculated with the samples mentioned above (10% w/v or 12% v/v) and benzene (0.2 mM) and different electron acceptors (nitrate, sulfate and chlorate (10 mM)). Heat killed controls and batches without addition of inoculum are also examined in order to see whether biodegradation is related to the inoculum.

PH, VVB and Rep are all present in the polluted sediment, soil and sludge collection of the Sub-department of Environmental Technology (WUR). These inocula are used for preliminary research because these were readily available. Furthermore, the sediment and soils contained PAH and petroleum hydrocarbons (8) and PH was successfully used as inoculum for anaerobic toluene degradation with AQDS as electron acceptor (6). Besides benzene measurements (GC) also the electron acceptors are also analyzed with an anion HPLC (Dionex).

The results, till now, indicate that benzene is still not degraded (batches are running for different periods). However, measurement of the electron acceptor show that in the PH incubation nitrate and chlorate reduction occurs and in the Rep batches nitrate reducing activity is present. Due to the fact that other substrates (hydrocarbons) are present, it is logic that these processes are occurring. The chlorate and nitrate in these batches were completely consumed and were readded (20 mM) at day 51.

Chlorate reducing organisms

Since the first two isolated strains that can degrade benzene anaerobically were chlorate reducing organisms (7) we are testing chlorate reducing strains AW-1 (29), GR-1 (22) and MA-1 (newly isolated strain and not described yet). These strains were kindly provided by Athur Wolterink and are able to oxidize acetate coupled with chlorate reduction. Strain MA-1 and strain GR-1 are also able to utilize nitrate and perchlorate as electron acceptor with acetate as electron acceptor.

The three strains are exposed to benzene (0.1 and 1.0 mM) in mineral medium (29) and the electron acceptors they can use (chlorate, perchlorate or nitrate, 10 mM). Similar experiments are also conducted in which acetate (5 mM)

is added together with benzene (0.5 mM) in order to have already sufficient biomass available. Furthermore, these strains are also tested to degrade 2-, 3-, 4-chlorobenzoate and benzoate (1 mM) together with the different electron acceptors (10 mM). These experiments are conducted because the isolated strain RCB, which is able to degrade benzene anaerobically, was isolated on 4-chlorobenzoate (7).

The results of these experiments show no degradation of all the compounds tested after a period of more than 100 days. The results indicate that the chlorate reducing strains AW-1, Gr-1 and MA-1 that were isolated on acetate, have only the ability to degrade substrates that easily can be mineralized.

Soil, groundwater and column material with benzene degrading activity
Soil from NAM site and groundwater from Flebo site were kindly provide by TNO and tested on their ability to degrade benzene anaerobically. Previous batch experiments with Flebo (11) and Nam (2) showed that these inoculum sources could degrade benzene with nitrate as electron acceptor. Therefore, these inoculum sources are used to start anaerobic benzene biodegradation experiments under nitrate reducing conditions. Similar batch experiments are running with column material from Bioclear BV in Groningen. Bioclear had a soil column running in which benzene was degraded under sulfate reducing condition (24). The preliminary results from these different incubations show no biodegradation of benzene after around 50 days.

Enrichments Anna Ivanova, Peter Middeldorp and Melike Balk
Batch cultures, that were present in the collection of the laboratory of microbiology from (former) employees who worked on benzene (Anna and Peter) and toluene (Melike) degradation, are tested on their ability to degrade benzene (0.1-0.2 mM) under nitrate reducing (10 mM, Annna and Melike) and methanogenic conditions (Peter). The first results of these batch cultures show no biodegradation of benzene.

4 Future Research

As mentioned in the results none of the batch cultures that are incubated degrade benzene. As summarized in Table 2 lag phases for biodegradation of benzene can be very long up to even one year. Therefore, patience has to be taken but due to these long periods it would be nice to have active cultures to do further research such as proposed in the project proposal, isolation, phylogenetic and physiologic characterization. Different ways can be followed to get active cultures.

Enrichment cultures
Different enrichments that degrade benzene anaerobically are present over the world. We are trying to get these to our laboratory at the moment. The enrichment culture (benzene degrading nitrate-reducing culture), that Anna Ivanova enriched, is still present in Moscow and is frequently transferred to fresh medium and is still active. Furthermore, in Mexico there are also cultures present that we are trying to get.

A chemostat culture is present at TNO-MEP that degrades benzene under denitrifying conditions. Due to the fact that TNO-MEP is applying for a patent, this culture cannot be used at the moment. It is expected that this patent is submitted in October. After this, a mutual agreement between TNO and WUR can be signed for the use and application of this culture by the WUR. Furthermore, a batch culture is present at TNO-MEP that degrades benzene slowly under chlorate reducing conditions. This culture will be collected in the near future.

Column experiments
An approach to obtain biomass that can degrade benzene anaerobically is via continuous experiments. Continuous refreshing of medium can enhance growth of microorganisms. We will start anaerobic columns with sediment and soil material from different polluted sites and try to find anaerobic biodegradation of benzene.

Try other inoculum and different electron acceptors
An approach is to investigate other polluted inoculum sources and their ability to degrade benzene. This can be done together with other electron acceptors like humic like substance AQDS, chlorate or manganese (IV). The literature showed that inoculum sources that have been long time exposed to benzene can rapidly degrade benzene

(28). Both electron acceptors AQDS and manganese (IV) are able to degrade toluene under anaerobic conditions (6, 13) and chlorate reducing organisms are the first isolated organisms that can degraded benzene anaerobically (7).

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